

CHARACTERIZATION OF PROTEINS AND OTHER MACRO-  
MOLECULES BY AGAROSE GEL CHROMATOGRAPHY

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Running titel: Agarose GPC of proteins



## SUMMARY

A number of proteins and proteoglycans were chromatographed on a Sepharose 4B column at various ionic strengths and after application of various amounts of samples.  $K_{av}$  was negligibly affected by the chromatographic conditions. When  $K_{av}$  was plotted versus Stokes' radius it was found that molecules with different frictional ratios followed different relationships. These results are discussed in relation to the effects of molecular asymmetry described in a recent paper (Nozaki et al., Biochemistry 15 (1976), 3884-3890). Physical parameters for Sepharose 4B and 6B gels were calculated from chromatography of two protein mixtures.

## INTRODUCTION

Gel chromatography separates molecules with different radii<sup>1</sup>. The relation between molecular radius and elution volume has been used to determine the size of proteoglycan fragments after calibration of a Sepharose gel with proteins<sup>x</sup> of known size<sup>2</sup>. This method was used for characterization of corneal proteoglycans<sup>3</sup> and fragments of skeletal proteoglycans<sup>4</sup>. It was found, however, that the calibration proteins did not follow the predicted relations; the deviation was largest for asymmetric proteins. Therefore, it was considered necessary to investigate how the elution volume of macromolecules depends on Stokes' radius, frictional ratio, sample mass, ionic strength and differences between various batches of agarose gels. The results, which are partly inconsistent with some published reports discussed below but confirm recent data from Tanford's group<sup>5</sup>, are discussed in the present paper; some data were preliminary discussed at an ARVO meeting<sup>6</sup>.



## THEORY

The distribution coefficient  $K_d$  shows the fraction of the solvent volume inside the gel grains that is accessible to the sample<sup>7</sup> while  $K_{av}$  is equal to the fraction of the total gel grain volume that is accessible to the sample<sup>1</sup>. The partial specific volume of agarose is 0.6 ml/g<sup>8</sup>. Thus, for Sepharose 4B (nominal agarose concentration 4 % w/v)  $K_{av}$  equals  $0.98K_d$  while for Sepharose 6B (nominal agarose concentration 6 % w/v),  $K_{av}$  equals  $0.96K_d$ .

Stokes' radius ( $r_s$ ) for a molecule is defined as the radius of a hypothetical sphere which in diffusion encounters the same frictional coefficient ( $f$ ) as the real molecule. According to Stokes' law<sup>9</sup> the relationship between the frictional coefficient and the radius of a sphere is

$$f = 6\pi\eta r_s \quad (1)$$

where  $\eta$  is the viscosity of the solvent. Stokes' radius has also been used as designation for the radius of the "equivalent sphere" with the same properties as the molecule in gel chromatography or viscometry. The differences between the various types of Stokes' radii have been extensively discussed by Tanford and coworkers<sup>5,10-12</sup>. In the present paper,  $r_s$  always refers to Stokes' radius as defined by eqn 1. Stokes'-Einstein's equation, which is derived from Stokes' law (eqn 1), gives the basis for calculation of  $r_s$  from the diffusion coefficient ( $D^0$ ):

$$r_s = RT/6\pi N\eta D^0 \quad (2)$$

where  $R$  is the gas constant,  $T$  the absolute temperature, and  $N$  Avogadro's number. If  $r_s$  is expressed in nm, if  $D_{20,w}^0$  is expressed in  $\mu\text{m}^2/\text{s}$  and if the viscosity for water at  $20^\circ$  and the temperature 293 K are inserted in eqn 2, it will be reduced to

$$r_s = 214/D_{20,w}^0 \quad (3)$$

Laurent and Killander<sup>1</sup> have shown that  $K_{av}$  for a spherical molecule which is partly included in a chromatographic gel is related to  $r_s$  by

$$K_{av} = e^{-\pi L(r_s + r_r)^2} \quad (4)$$

which can be rearranged to<sup>13</sup>

$$(-\ln K_{av})^{1/2} = (\pi L)^{1/2}(r_s + r_r) \quad (5)$$

where  $L$  is the concentration of fibers in the gel grains (in this investigation the concentration of agarose fibers expressed in  $\text{m fiber}/\text{m}^3$  gel) and  $r_r$  the radius of the fibers.

In some cases, the frictional ratio ( $f/f_0$ ) for a protein was calculated from other literature data using the equation<sup>14</sup>

$$f/f_0 = 2.89 \times 10^{-3}/D(M\bar{v})^{1/3} \quad (6)$$

where  $M$  is the molecular weight and  $\bar{v}$  the partial specific volume.

## EXPERIMENTAL

### Materials

Proteins for calibration came from Sigma Chemical Company, St., Louis, Mo., USA; Calbiochem AG, Lucerne, Switzerland; and Serva Feinbiochemica GmbH & Co, Heidelberg, GFR (see Table I for details; this table also contains physical data for the proteins). Procedures described elsewhere were used for preparation of proteoglycans from bovine corneal stroma<sup>39</sup> (designated cornea-50P and cornea-70P<sup>39</sup>) and proteoglycan aggregates from bovine nasal cartilage<sup>28</sup>. Tritated water was from Packard Instrument Company, Inc., Downers Grove, Ill., USA. Sepharose gels were from Pharmacia Fine Chemicals, Uppsala, Sweden.

### Samples for gel chromatography

Solutions of proteins (0.5 and 1.0 mg/ml), corneal proteoglycans (0.75 and 1.5 mg/ml) and cartilage proteoglycans (1.0 mg/ml) were made. Fibrinogen was dissolved in 1 M NaCl-0.05 TrisHCl pH 7.0. All other proteins were dissolved in 0.5 M TrisHCl (pH 7.0). Cornea-50P was dissolved in 4 M guanidinium chloride-0.05 M sodium acetate (pH 5.8) and dialyzed extensively against the elution buffer since it is difficult to dissolve it in the absence of denaturing agents. Cornea-70P was dissolved directly in the elution buffer. l-Glutamate dehydrogenase was obtained as a suspension (20 mg protein/ml). Two portions were diluted twentyfold and fortyfold, respectively, with



0.5 M Tris-HCl buffer (pH 7.0) and dialyzed against the same buffer in order to get solutions with protein concentrations about 1 mg/ml and 0.5 mg/ml, respectively. Crude  $\alpha$ -globulin solutions with protein concentrations 3 and 6 mg/ml were made; this makes the  $\alpha_2$ -macroglobulin concentration very approximately 0.5 and 1.0 mg/ml, respectively. Solutions of glucuronolactone (0.1 mg/ml) and triated water (12 % v/v) were made with distilled water.

To simplify the calibration procedure, two mixed solutions with selected proteins in Tris buffer were made. Protein solution A contained cartilage proteoglycan aggregates (1 mg/ml), peroxidase (0.5 mg/ml) and urease (0.5 mg/ml); protein solution B contained thyroglobulin (0.5 mg/ml) and serum albumin (0.5 mg/ml).

The sample volume applied to the columns was 400  $\mu$ l. Thus, the mass of sample applied to the column was for proteins 0.2 or 0.4 mg, for corneal proteoglycans 0.3 or 0.6 mg and for cartilage proteoglycan aggregates 0.4 mg.

#### Gel chromatography

One column of Sepharose 4B and one of Sepharose 6B were used. Both were packed in glass tubings with internal diameters of 0.6 cm. A peristaltic pump maintained the flow rate at 2.4 ml/h through both columns and 0.58 ml fractions were collected. If the columns were packed with a slightly higher buffer flow than used for elution, the system was very stable. The Sepharose 4B column was used for about



one hundred chromatographic runs during more than two years without any detectable changes in  $V_o$  (the void volume) or  $V_i$  (the solvent volume inside and outside the grains in the gel). This column had a length of 133 cm,  $V_i$  38.5 ml and  $V_o$  13 ml. The Sepharose 6B column had a bed length of 145 cm,  $V_i$  42 ml and  $V_o$  16 ml.

Two elution buffers were used: 0.15 M NaCl-5mM diemal sodium buffer - 0.02 %  $\text{NaN}_3$  (pH 7.0) and 1 M NaCl-5mM diemal sodium buffer 0.02 %  $\text{NaN}_3$  (pH 7.0). Diemal buffers do not interfere with the Folin procedure in contrast to Tris or phosphate buffers. The bacteriostatic agent sodium azide must be deleted when the carbazole method is used for analysis of column effluents.

#### Analytical procedures

Automatic methods<sup>29</sup> were used for analysis of proteins<sup>30</sup> and glucuronolactone<sup>31</sup> in column effluents. Tritiated water was measured by liquid scintillation counting.

The elution volume for a sample was considered to be the elution volume of the fraction with the highest concentration. However, if the peak was asymmetric and the second highest fraction had a concentration over 90 % of that in the fraction with the peak concentration, the midpoint between the two fractions was considered as the elution volume (Fig. 1). Tris buffer often eluted as a peak with several top fractions within the same range of concentration; the midpoint of this plateau was used as a marker for  $V_i$  (Fig. 1). Urease gave on-

ly one significant peak in contrast to other preparations<sup>13</sup>. This peak was considered to correspond to the main fraction described by Sumner et al.<sup>26</sup>  $\alpha_2$ -Macroglobulin was part of a crude  $\alpha$ -globulin preparation; the  $\alpha_2$ -macroglobulin peak was distinguished from the other  $\alpha$ -globulins since it has larger molecular size and thus lower  $K_{av}$ .

TABLE I.

DATA FOR PROTEINS USED FOR CALIBRATION OF AGAROSE

| Protein                                           | Commercial designation | Stokes radius<br>(nm) | Frictional ratio<br>( $f/f_0$ ) | Molecular weight     | References      |
|---------------------------------------------------|------------------------|-----------------------|---------------------------------|----------------------|-----------------|
| 1. Peroxidase<br>(horse-radish)                   | Sigma<br>P-8250        | 3.03                  | 1.36                            | 39 800               | 15              |
| 2. Serum albumin<br>(bovine)                      | Sigma<br>A-4503        | 3.48                  | 1.30                            | 65 400               | 16              |
| 3. Transferrin<br>(human)                         | Sigma<br>T-2252        | 3.55 <sup>a</sup>     | 1.26 <sup>b</sup>               | 76 600               | 17              |
| 4. Catalase<br>(bovine liver)                     | Sigma<br>C-40          | 5.12                  | 1.24                            | 243 000              | 18              |
| 5. Urease<br>(jack bean)                          | Sigma<br>U-0376        | 6.52                  | 1.22 <sup>b</sup>               | 520 000 <sup>c</sup> | 19              |
| 6. Apoferritin<br>(horse spleen)                  | Calbiochem<br>17836    | 6.73 <sup>a</sup>     | 1.32                            | 460 000              | 20              |
| 7. L-Glutamate<br>dehydrogenase<br>(bovine liver) | Sigma<br>G-2501        | 7.22 <sup>a</sup>     | 1.58 <sup>b</sup>               | 316 000              | 21 <sup>d</sup> |
| 8. $\alpha$ -casein<br>(cow milk)                 | Sigma<br>C-3883        | 7.37                  | 2.25                            | 121 800              | 22              |
| 9. Thyroglobulin<br>(bovine)                      | Sigma<br>T-1001        | 8.58                  | 1.49                            | 669 000              | 23              |
| 10. $\alpha_2$ -Macroglobulin<br>(human)          | Serva<br>22390         | 8.87                  | 1.43                            | 820 000              | 24              |
| 11. Fibrinogen<br>(human)                         | Calbiochem<br>341576   | 10.7                  | 2.34                            | 340 000              | 25              |

<sup>a</sup>Calculated from Svedberg's equation<sup>9</sup> and eqn 2.<sup>b</sup>Calculated from eqn 6.<sup>c</sup>Calculated from Svedberg's equation<sup>9</sup> using a  $\bar{v}$  value from ref. 26<sup>d</sup>Data on  $s_{20,w}^0$  and  $\bar{v}$  from ref. 27.

## RESULTS

### Calibration of $V_o$ and $V_i$ .

Proteoglycan aggregates from bovine hyaline cartilage are eluted in the void volume of Sepharose 2B columns<sup>32</sup> and can thus be used to indicate  $V_o$  (Fig. 1). Tritiated water and glucuronic acid have been widely used to indicate  $V_i$ . Tris has the same elution volume (Fig. 1) and was preferred as  $V_i$  indicator since it was detected by the Folin procedure used for analysis of proteins in column effluents.

### Chromatography of individual proteins and proteoglycan samples on Sepharose 4B.

Each protein was chromatographed separately on the Sepharose 4B column. Three runs were made with 0.2 mg protein eluted with 0.15 M NaCl buffer (Fig. 2), one run with 0.4 mg protein eluted with 0.15 M NaCl buffer (Table II) and one run with 0.4 mg protein eluted with 1.0 M NaCl buffer (Table II). Similar runs were made with corneal proteoglycans (Table II).  $\alpha$ -Casein occurs in different types of aggregates depending on buffer composition<sup>33</sup>. Therefore a control experiment was made with the buffer used by Sullivan et al.<sup>22</sup> (0.08 M NaCl, 0.02 M diemal-Na, pH 7.8). The sample was dissolved in and eluted with this buffer. The  $K_d$  value obtained (0.80) is within the range of the other  $K_d$  values for  $\alpha$ -casein (Table II).

### Chromatography of calibration solutions on Sepharose 4B and 6B.

Chromatography of calibration solutions A and B on Sepha-



rose 6B is shown in Fig. 1. Values of  $(-\ln K_{av})^{1/2}$  from these runs were plotted versus Stokes' radii (Fig. 3). For comparison, the calibration curves obtained by Laurent<sup>8</sup> with Ficoll samples are also shown in the graph. The elution volume on Sepharose 4B of the individual proteins in the calibration solutions are in all cases the same as those obtained when the proteins are chromatographed separately. The equation for the regression line in Fig. 2 is  $(-\ln K_{av})^{1/2} = 0.061 r_s + 0.32$ . The equation for the regression line which shows chromatography of protein solutions A and B on Sepharose 4B (Fig. 3) is  $(-\ln K_{av})^{1/2} = 0.064 r_s + 0.31$ . The differences between the equations are small. It is thus sufficient to use solutions A and B for calibration of 4-6 % agarose columns.

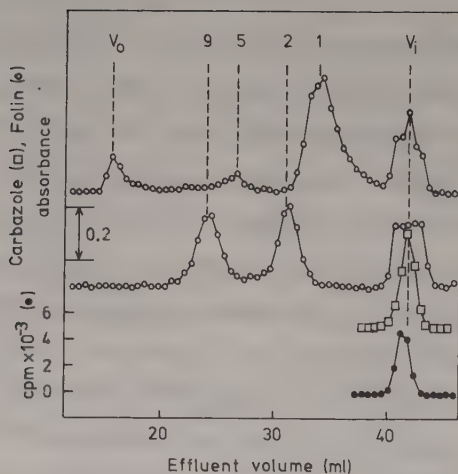


Fig. 1. Calibration of the Sepharose 6B column eluted with the 0.15 M NaCl buffer. The curves show from the top downwards protein solution A; protein solution B; glucuronic acid lactone; and tritiated water. The numbers refer to Table I. The urease (5) sample (total 0.2 mg) contained not only urease but also some salt; this probably explains the low absorbance given by this protein.

## DISCUSSION

### Factors of importance for the elution volume of proteins and proteoglycans.

Sample mass. Increased amount of sample may increase the  $K_{av}$  value of the sample<sup>34</sup>. The high sensitivity of the Folin method makes it possible to use small amounts of sample and no significant effect of the sample mass on  $K_{av}$  could be detected within the mass range used (Table II).

Ionic strength. At low ionic strength the volume of polyanions such as proteoglycans, glycoproteins and proteins increases due to repulsion between negative charges. Moreover, Sephadex and Sepharose gels have carboxyl and sulphate groups that cause ion-exclusion effects<sup>35</sup> when low ionic strength buffers are used for elution. Therefore, Crone<sup>35</sup> suggests that elution buffers for Sepharose columns should have an ionic strength not less than 0.2. Bovine corneal keratan sulfate proteoglycans e.g. are completely excluded from Sepharose 4B at pH 4.0 and ionic strength 0.001 but only partly excluded at higher ionic strength (unpublished). For the proteins and proteoglycans at pH 7, two ionic strengths, 0.16 and 1.0, were chosen. No significant differences in  $K_{av}$  were obtained (Table II). It thus seems that physiological ionic strength could be used for chromatography of the proteins and proteoglycans investigated.

Stokes' radius. It follows from eqn 5 that  $(-\ln K_{av})^{1/2}$  is a linear function of  $r_s$ . However, most of the  $(-\ln K_{av})^{1/2}$  values in Fig. 2 deviate from the regression line despite

TABLE II

EFFECT OF SAMPLE MASS AND IONIC STRENGTH OF ELUTION BUFFER ON ELUTION VOLUME OF SAMPLE

The first column of  $K_d$  values for proteins are those used in Figs. 2 and 4.

| Protein                      | $K_d$                 |                     |                    |
|------------------------------|-----------------------|---------------------|--------------------|
|                              | Sample mass 0.2 mg    | Sample mass 0.4 mg  | Sample mass 0.4 mg |
|                              | Ionic strength 0.16   | Ionic strength 0.16 | Ionic strength 1.0 |
|                              | Average of three runs | One run             | One run            |
| Peroxidase                   | 0.82                  | 0.82                | 0.82               |
| Serum albumin                | 0.75                  | 0.75                | 0.74               |
| Transferrin                  | 0.74                  | 0.73                | 0.74               |
| Catalase                     | 0.71                  | 0.70                | 0.72               |
| Urease                       | 0.59                  | 0.59                | 0.60               |
| Apo ferritin                 | 0.61                  | 0.60                | 0.59               |
| l-Glutamate<br>dehydrogenase | 0.63                  | 0.63                | 0.63               |
| $\alpha$ -Casein             | 0.81                  | 0.76                | 0.78               |
| Thyroglobulin                | 0.50                  | 0.50                | 0.48               |
| $\alpha_2$ -Macroglobulin    | 0.53                  | 0.51                | 0.55               |
| Fibrinogen                   | 0.50                  | 0.49                | 0.49               |

| Proteoglycan                                | $K_d$               |                     |                    |
|---------------------------------------------|---------------------|---------------------|--------------------|
|                                             | Sample mass 0.3 mg  | Sample mass 0.6 mg  | Sample mass 0.3 mg |
|                                             | Ionic strength 0.16 | Ionic strength 0.16 | Ionic strength 1.0 |
|                                             | One run             | One run             | One run            |
| Cornea-50P,<br>peak C<br>(ref. 39, Fig. 4b) | 0.29                | 0.29                | 0.31               |
| Cornea-70PA <sup>a</sup>                    | 0.33 (0.31)         | (0.32)              | (0.29)             |
| Cornea-70PB <sup>a</sup>                    | 0.56 (0.54)         | (0.55)              | (0.55)             |

<sup>a</sup>Cornea-70PA and cornea-70PB refer to the first and second peak that cornea-70P shows when chromatographed on Sepharose 4B. Data within brackets were obtained with a batch of Sepharose 4B different from the one used for other experiments presented in this paper.



the good reproducibility of the  $K_{av}$  values. It is probable that inaccurate diffusion constants are partly responsible for the scatter of the points in Fig. 2. The role of asymmetry must, however, also be considered since the two asymmetric proteins investigated ( $\alpha$ -casein and fibrinogen) show much lower  $(-\ln K_{av})^{1/2}$  values than expected.

Asymmetry. If the proteins are grouped according to their  $f/f_0$  values, it seems that those with higher frictional ratios have lower  $(-\ln K_{av})^{1/2}$  values than expected from those with lower frictional ratios (Fig. 2). It may be concluded that molecular asymmetry causes retardation at agarose gel chromatography. This effect of asymmetry may be obscured by the facts that  $f/f_0$  is a function of both shape and hydration and that many diffusion constants probably are inaccurately determined.

The observations in this investigation on the role of molecular asymmetry in gel chromatography are not compatible with some earlier findings. Siegel and Monty<sup>13</sup> found that bovine fibrinogen ( $f/f_0 = 2.35$ ) was perfectly adapted to the regression line for globular proteins in a plot of  $(-\ln K_{av})^{1/2}$  versus Stokes' radius for Sephadex G-200 eluted with 0.04 M phosphate buffer-5mM EDTA (pH 8.0). Demassieux and Lachance<sup>36</sup> also used bovine fibrinogen and globular proteins for calibration of a Sepharose 6B column. They found a linear relationship between  $\log r_s$  and  $K_d$ . However, they used a 0.1 M phosphate buffer (pH 7.0) containing the reducing agent dithiothreitol (1 mM) and EDTA

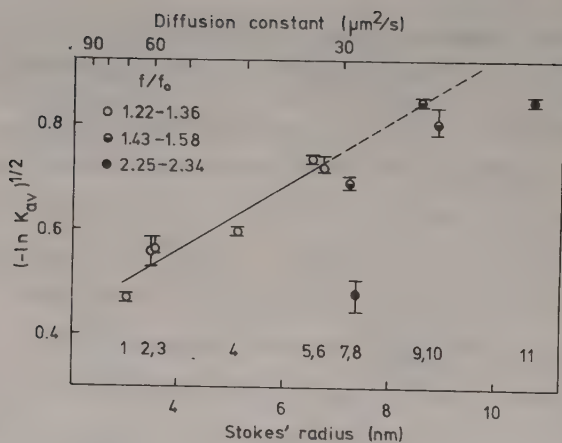


Fig. 2. Chromatography of proteins on the Sepharose 4B column eluted with the 0.15 M NaCl buffer. The horizontal bars indicate the range and the circles the average of  $(-\ln K_{av})^{1/2}$  calculated from three runs of each protein (0.2 mg). The straight line is a best least squares line for proteins with  $f/f_0$  values in the range 1.22-1.36. The numbers on the proteins refer to Table I.

(1 mM) which may influence the conformations of the proteins. These latter authors also state that globular and asymmetric proteins fit to a common regression line if  $\log \left( (f/f_0) M^{1/3} \right)$  is plotted versus  $K_d$ . Therefore, the chromatographic data from Fig. 2 were plotted as  $\log \left( (f/f_0) M^{1/3} \right)$  versus  $K_d$  (Fig. 4). The scatter of points is large and the reliability of the empiric equations formulated by these authors seems limited. Elution buffers with reducing agents were not used in the present investigation since there is little reason to believe that the varying degree of unfolding caused by reduction in the absence of dissociating agents should make  $K_d$  a linear function of  $\log \left( (f/f_0) M^{1/3} \right)$ .

However, careful review of gel chromatographic data in the literature reveals several indications that molecular asymmetry may influence results in gel chromatography. Laurent and Killander<sup>1</sup> stressed that eqn 4 is derived for spherical particles only. The data of Warshaw and Ackers<sup>37</sup> suggest that molecular asymmetry may influence the gel chromatographic behaviour of proteins. Laurent et al.<sup>38</sup> state that asymmetric molecules penetrate polysaccharide gels more readily than globular molecules with equal Stokes' radius. Since  $K_{av}$  indicates the fraction of gel accessible for the molecular species this observation is consistent with higher  $K_{av}$  values for asymmetric molecules than for globular molecules with equal  $r_s$ . Tanford et al.<sup>12</sup> stated in 1974 that "no systematic studies using a mixture of globular and highly asymmetric mo-

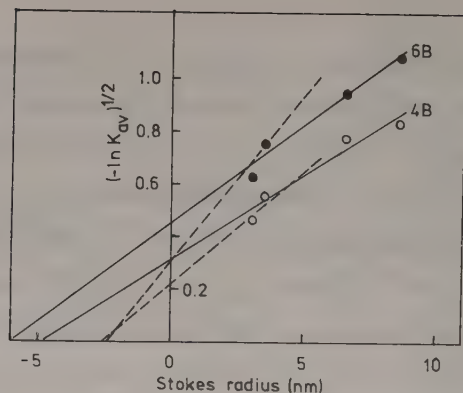


Fig. 3. Calibration of the Sepharose columns with protein solutions A and B. One single run was made with each solution on each column. Solid lines indicate the best least square lines for these runs; dashed lines show the corresponding lines obtained by Laurent<sup>8</sup> for 4 % and 6 % agarose, respectively.

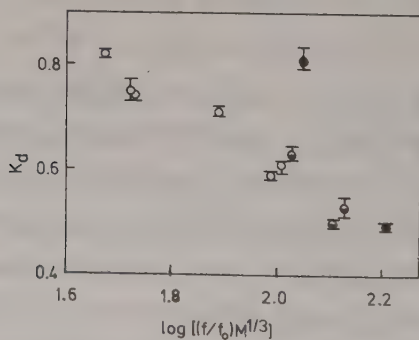


Fig. 4. The data from Fig. 2 plotted as suggested by Demassieux and Lachance<sup>36</sup>. See Fig. 2 for explanation of symbols. The proteins are in order from left (no 1, Table I) to right (no 11).

lecules have been carried out to determine whether partition in the gel is consistently responsive to the  $R_s$  Stokes' radius value based on either frictional coefficient or intrinsic viscosity". Recently, Nozaki et al.<sup>5</sup> described the retardation of fibrinogen and myosin and some denaturated proteins on Sepharose 4B. The present investigation corroborates these data on a pronounced retention of asymmetric proteins and previous data<sup>37</sup> on a slight retention of "globular" proteins with  $f/f_0$  in the range 1.4 - 1.6<sup>xx</sup>.

The role of molecular asymmetry has with few exceptions been overlooked in the interpretation of data from gel chromatography; still, there is a need for systematic investigations of the relations between molecular shape and exclusion from gels.

Differences between gels. Fig. 3 and Table III show some data on the Sepharose 4B and 6B gels compared to the data obtained by Laurent<sup>8</sup> from calibration of 4 % and 6 % pearlcondensed agarose gels with various fractions of Ficoll, a highly branched polysaccharide. The values for fiber radius, fiber volume and hydration are larger than those of Laurent and the values for fiber length are consequently smaller. This may reflect both differences in the nature of the calibration molecules (globular protein versus polysaccharides) and differences between various batches of agarose. Laurent compared human serum albumin with Ficoll and found a  $K_{av}$  for albumin equal to the  $K_{av}$  for Ficoll with the same  $r_s$ ; therefore it seems probable that Ficoll behaves



TABLE III

## DATA FOR THE AGAROSE GELS

| Gel type                       | Fiber radius<br>( $r_f$ ; nm) | Total length<br>of fibers<br>( $L$ ; $[m/m^3] \times 10^{-15}$ ) | Fiber volume<br>( $m^3$ fiber/ $m^3$<br>gel grains) | Hydration of<br>fibers<br>( $H_2O$ , % w/w) <sup>a</sup> |
|--------------------------------|-------------------------------|------------------------------------------------------------------|-----------------------------------------------------|----------------------------------------------------------|
| 4 % agarose                    |                               |                                                                  |                                                     |                                                          |
| Data from this paper           | 4.8                           | 1.3                                                              | 0.095                                               | 64                                                       |
| Data from Laurent <sup>8</sup> | 2.6                           | 2.4                                                              | 0.049 <sup>b</sup>                                  | 37 <sup>b</sup>                                          |
| 6 % agarose                    |                               |                                                                  |                                                     |                                                          |
| Data from this paper           | 5.9                           | 1.8                                                              | 0.20                                                | 73                                                       |
| Data from Laurent <sup>8</sup> | 2.4                           | 5.1                                                              | 0.092 <sup>b</sup>                                  | 48 <sup>b</sup>                                          |

<sup>a</sup>Calculated as g water per g of hydrated agarose fiber using 1.6 g/ml as the density for pure unhydrated agarose<sup>8</sup>.

<sup>b</sup>Data calculated from other data given by Laurent<sup>8</sup>.

like globular proteins. However, it is possible that large molecules do not follow eqn 4 strictly since they may be completely excluded from the regions around the branching points of the agarose network. The  $K_{av}$  values on 4 % agarose were in the range 0.5-0.8 for all samples in the present investigation (Table II) and in the range 0.6-0.85 for the Ficoll samples used by Laurent. Therefore, it is not possible to discuss the behaviour of molecules that elute close to  $V_0$  or  $V_i$ . It is assumed in eqn 4 that the gel consists of randomly distributed, linear, rigid rods with infinite length; deviations from this will lead to apparent thickening and shortening of the rods<sup>1</sup>. Thus, it is possible that the smaller molecules used by Laurent give more reliable data on the fiber radius, fiber volume and fiber length of agarose gels than the larger molecules used in the present investigation. This would partially explain the lower slope of the curves in Fig. 3 and the higher values for fiber volume and fiber radius obtained in the present investigation. This would also imply that it is a too large approximation to consider the agarose fibers to be of infinite length, without branching points.

However, there are undoubtedly also differences between different batches of agarose with the same nominal concentrations and this may explain the differences between the present investigation and Laurent's investigation. Laurent<sup>8</sup> and Nozaki et al.<sup>5</sup> found different properties for different batches of 4 % agarose from Pharmacia. Sepharose 2B is used

for determination of the aggregate/monomer ratio for cartilage proteoglycans since aggregates are completely excluded from the gel while monomers are partially included in the gel<sup>32</sup>. However, the same batch of proteoglycans chromatographed on different batches of Sepharose 2B gave different elution profiles with considerable variation in the amount of completely excluded material and thus different values for the aggregate/monomer ratio (D. Heinegård & S. Inerot, personal communication). It can be concluded that comparison of gel chromatograms from different batches of agarose gels must be made with caution and that there is a need for investigation of the relationship between  $K_{av}$  and  $r_s$  within the whole range of  $K_{av}$  values.

#### ACKNOWLEDGEMENT

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# FOOT-NOTES

<sup>x</sup>It should be pointed out that in the plot (Fig. 1) in the paper of Serafini-Fracassini et al.<sup>2</sup> the points have got wrong numbers; they should be numbered from right to the left in the graph.

<sup>xx</sup>An arbitrary but mostly accepted definition of globular proteins is proteins with  $f/f_0$  below 1.50 (ref. 24, p. C3).

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